Short Communication

**In vivo expression of genes in the entomopathogenic fungus *Beauveria bassiana* during infection of lepidopteran larvae**

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The entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuillemin is commercially available as a bioinsecticide. The expression of three genes previously identified to have a role in pathogenicity in *in vitro* studies was validated *in vivo* in three lepidopteran insects infected with *B. bassiana*. Expression of all three genes was observed in all the tested insects starting from 48 or 72 h to 10 d post infection corroborating their role in pathogenicity. We suggest that it is essential to test the expression of putative pathogenicity genes both *in vitro* and *in vivo* to understand their role in different insect species.

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# Article info

**Abstract**

The entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin has a host range of >700 insect species. Recent research indicates that *B. bassiana* is a generalist fungus with different isolates of this complex species showing no host specificity (Rehner and Buckley, 2005; Uma Devi et al., 2008). In the genome of *B. bassiana* (accession no: ADAH01000227), ~2000 genes have been implicated in pathogenicity (Xiao et al., 2012). Validation of these putative pathogenicity genes requires thorough insights into host-pathogen-interactions.

Our earlier *in vitro* study of *B. bassiana* cultures on cuticle extracts of different insect species and synthetic medium revealed 46 differentially expressed transcripts (Khan et al., 2007). Here, we validate *in vivo* expression of three of them: Subtilisin (Pr1H) and exocyst component (Sec15) genes reported to be directly involved in pathogenicity or to confer abiotic stress tolerance in other fungal species (Perinotto et al., 2014; Uma Devi et al., 2012) and a third transcript (EC391425) with unknown function (Khan et al., 2007). EC391425 was selected because it was expressed on the cuticular extracts of all tested insects but not on synthetic medium (Khan et al., 2007) compellingly suggesting its role in pathogenicity.

Larvae of three lepidopteran insects – the greater wax moth *Galleria mellonella* (Linnaeus), the cactus moth *Cactoblastis cactorum* (Berg) and the European grapevine moth *Eupoecilia ambiguella* (Hubner) for which *B. bassiana* was reported to be pathogenic (Gupta et al., 1994; Legaspi et al., 2010; Cozzi et al., 2013) were obtained from laboratory rearings at Geisenheim University. Third instar larvae (60 of each species) were treated with an aqueous (with 0.01% Tween 80) conidial suspension (2×10⁷ conidia ml⁻¹) obtained from a 14 d culture of *B. bassiana* ITCC4688 on SDY medium. Mock larvae treated with 0.01% Tween 80 served as controls.

For qPCR, insects were collected after infection at 24, 48 and 72 h, 7 d and 10 d (Fig. 1) and stored at −80 °C. At each time, two pools (biological replicates) each of five larvae were collected. Mock treated insects were also collected and labeled as 0 h. RNA was extracted from insects with Trizol (Invitrogen, Karlsruhe, Germany) and reverse transcribed using DYNAmo™ cDNA synthesis kit (Finnzymes Espoo, Finland) according to manufacturer’s protocol. qPCR was done following MIQE guidelines (Bustin et al., 2009) on iQ5 real-time PCR system (Bio-Rad, Munich, Germany) using DyNAmo™ Color Flash SYBR® Green Kit (Finnzymes Espoo, Finland). The expression of three genes previously identified to have a role in pathogenicity in *in vitro* studies was validated *in vivo* in three lepidopteran insects infected with *B. bassiana*. Expression of all three genes was observed in all the tested insects starting from 48 or 72 h to 10 d post infection corroborating their role in pathogenicity. We suggest that it is essential to test the expression of putative pathogenicity genes both *in vitro* and *in vivo* to understand their role in different insect species.

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Finland) according to manufacturer’s instructions. β-tubulin and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were used as reference genes. Primers for selected genes were designed using Primer 3 software (Rozen and Skaletsky, 2000) (Table 1). PCR amplifications were done in a volume of 20 μl with 2 μl cDNA (1:10 diluted) as template, 10 pmol of each primer and 10 μl DyNAmo master mix (Finnzymes Espoo, Finland). A two step cycling program was used consisting of 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s, annealing for 30 s at a temperature suitable for each gene (Table 1) and extension at 72 °C for 40 s. A minimum of two independent technical replicates for each cDNA sample were done with each primer pair. Quantification cycle (Cq) values were calculated using iQ5 ver. 2 (BioRad, Munich, Germany). Quantification of gene expression was done using qBase. ANOVA statistical analysis was done at http://www.assistat.com/indexi.html.

In vivo expression of the three selected genes was observed in samples obtained 48 h after B. bassiana treatment from G. mellonella and E. ambiguella and 72 h after infection from C. cactorum (Fig. 1). Expression of Pr1H was found to gradually increase during the progression of infection and was significantly up-regulated at conidiospore stage (10 d) compared to other stages in all three insect species (G. mellonella: P < 0.001, F = 245.6; E. ambiguella: P < 0.001, F = 130.12, DF = 3; C. cactorum: P = 0.0025, F = 78.87, DF = 3) (Fig. 1a). In G. mellonella, expression levels of Pr1H were found to be lower at 48 h than at later stages (P < 0.001, F = 245.6, DF = 3). Expression of Sec15 was found to remain constant during all stages in E. ambiguella (P > 0.100, F = 6.591, DF = 3) and C. cactorum (P > 0.100, F = 9.5251, DF = 3), while in G. mellonella, a significantly higher level of expression was observed at 10 d compared to the earlier samples (P = 0.0041, F = 26.83, DF = 3) (Fig. 1b). Expression

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Table 1
The details of primers used for qPCR analysis for studying expression of three selected genes during pathogenesis of Beauveria bassiana.

<table>
<thead>
<tr>
<th>Gene/accession no.</th>
<th>Forward primer (5’–3’)</th>
<th>Reverse primer (5’–3’)</th>
<th>Ta (°C)</th>
<th>PCR efficiency (%)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-tubulin/DQ090731</td>
<td>TCGAGGGCATGATTGCTAC</td>
<td>GCGAGGGATACAGTGTCGTC</td>
<td>60</td>
<td>90.20</td>
<td>121</td>
</tr>
<tr>
<td>GAPDH/GT897067</td>
<td>GATGGATATTTATCCATGTC</td>
<td>CTGAGGATATCGTGTCGTCG</td>
<td>56</td>
<td>104.80</td>
<td>141</td>
</tr>
<tr>
<td>Exocyst component</td>
<td>TTAAGAGACGGCTACGTCGAG</td>
<td>ACCAGACAGATCTCTAGAGAA</td>
<td>60</td>
<td>107</td>
<td>160</td>
</tr>
<tr>
<td>pr1H/EC391425</td>
<td>AGGCTATACATGACTGCTACG</td>
<td>TGGGAAACCTTCTGATGCTTA</td>
<td>60</td>
<td>109.50</td>
<td>98</td>
</tr>
<tr>
<td>pr1H/EC391420</td>
<td>TAGTAGGAGCCGTACCCACT</td>
<td>CTTGCGCTGATTTTACGCGG</td>
<td>60</td>
<td>98</td>
<td>120</td>
</tr>
</tbody>
</table>

Fig. 1. Relative expression levels of (a) subtilisin like protease Pr1H; (b) exocyst component Sec15; (c) EC391425 transcript in Beauveria bassiana during pathogenic cycle on third instars larvae of three species of Lepidoptera. The expression was studied at five time points post infection (24 h, 48 h, 72 h, 7 d, 10 d) along with mock treated samples (0 h). Values represent mean ± SE of two technical and two biological replicates. Within the same lepidopteran species, means followed by distinct letters differ significantly at 5% probability. Images of progression of infection of third instars larvae of Lepidoptera species were represented at the respective time points on the Scale X.
of EC391425 was different in the three insects. In G. mellonella, expression was significantly down-regulated 72 h and showed maximum expression at 7 d (Fig. 1c). Expression was variable in G. mellonella at different times. In E. ambiguella, expression remained constant from 48 h onwards (Fig. 1c). In C. cactorum, significantly lower level of expression was observed in 10 d samples compared to others (P = 0.003, F = 60.72, DF = 3).

All the three genes selected based on results of in vitro assays (Khan et al., 2007) were found up-regulated in vivo in all the insects tested. Culturing the fungus on insect cuticle extracts in in vitro studies (Khan et al., 2007) simulated an initial infection stage which includes the establishment of conidial contact with the insect cuticle, its germination and penetration. In in vitro experiments, the Pr1H and Sec 15 genes were not up-regulated when B. bassiana was cultured on the cuticular extract of the lepidopteran insect Spodopera littura (Khan et al., 2007). In the present in vivo assays, expression of both genes was not evident until 48 h post infection. Fang and Bidocha (2006) reported that pathogenicity gene expression could not be detected in early stage of infection due to low fungal biomass in the insect. qPCR can detect very low gene expression levels (Bustin, 2000; Pfaffl and Hageleit, 2001; Schmittgen et al., 2000). Therefore, the lack of detection of all three genes in samples collected 24 h is likely due to a lack of expression of these genes and not due to low abundance of fungal biomass. This hypothesis however, needs to be proved by defining a detection limit of B. bassiana propagules in the given qPCR assay.

Pr1H is an endocellular subtilisin (Baga et al., 2004) and was reported to be expressed on sporulating cultures of B. bassiana growing on chitin (Cho et al., 2006). In our in vivo assays, this gene was highly expressed during the sporulating stage on the insect cadaver. It was also not expressed in in vitro studies on the lepidopteran insect S. littura. The expression profile of Pr1H thus suggests that in lepidopteran insects it’s accumulation in sporulating mycelia or conidia emerging on the insect cadavers ‘primes’ them for next round of infection. Sec 15 gene was found to be expressed at constant levels throughout the infection cycle in all three lepidopteran insects tested in vivo. Sec 15, a component of exocyst complex being essential for secretary pathway (Vega and Hsu, 2001) might facilitate secretion of enzymes and toxins to overtake the host and utilize it as a substrate (Beys-da-Silva et al., 2014). However, in previous in vitro assays, Sec 15 was not expressed on cuticular extracts of S. littura and Epilachna vigintioctopunctata but expressed on extracts of Aphis craccivora and Periplaneta americana (Khan et al., 2007). Thus the stage of expression of the Sec15 gene in B. bassiana differs in different insect species. Expression of EC391425 with a so far unknown function was not observed on synthetic medium but expressed on insects both in vitro (Khan et al., 2007) and in vivo insistently implying its role in pathogenicity of B. bassiana. We are presently working on determining its functional role.

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